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METHOD FOR INDUCING VIRAL RESISTANCE INTO A PLANT

Field of the invention

The present invention is related to a method for inducing viral resistance into a cell and a plant, especially BNYVV-resistance into a sugar beet cell and plant.

Background of the invention and state of the art

The widespread viral disease of the sugar beet plant (*Beta vulgaris*) called Rhizomania is caused by a benyvirus, the beet necrotic yellow vein virus (BNYVV) (23, 24) which is transmitted to the root of the beet by the soilborne fungus *Polymyxa betae* (25).

The disease significantly affects acreages where the sugar beet plant is grown for industrial use in Europe, USA and Japan and is still in extension in several places in Western Europe (26, 27). As there exists no practical method to effectively control the spread of the virus at a large scale by chemical or physical means (28), neither in the plants nor in the soil, the main focus has been to identify natural sources of resistance within the sugar beet germplasm and to develop by breeding, varieties of sugar beet plants expressing the resistance genes. A variety of such tolerance genes to the virus have been identified and, some have been successfully used in the breeding of commercial sugar beet varieties (29, 30, 31).

Only the use of BNYVV-resistant or tolerant varieties will enable farmers to grow sugar beet plants in BNYVV-infected areas where the sugar beet plant is an essential component of the crop rotation and contributes significantly to the grower's income.

A number of detailed studies have shown that a difference in susceptibility to the BNYVV-infection among sugar beet genotypes or varieties, generally reflect difference in the diffusion or translocation of the virus in the root tissues (32).

However, there are still few reports which indicate clearly that the tolerance genes, even from differing sources of sugar beet germplasm or wild relatives germplasm (33), would provide distinct mechanisms of resistance. Such a situation would represent a more manageable situation to design long lasting BNYVV-resistance strategies.

Since 1986, a number of reports and publications have described the use of isolated viral gene sequences expressed in plants to confer a high level of tolerance against the virus or even to confer a broad spectrum type of resistance against a number of related viruses (34, 35, 36). One of the most documented viral resistance strategy based on genetic engineering, in many cultivated species such as potato, squash, cucumber or tomato, is the use of the viral gene sequence which under the control of plant regulatory elements, encodes the coat-protein of the target virus (37).

However, for coat-protein mediated resistance, the expression of a certain level of resistance in the transgenic plant might be attributed to different mechanisms such as RNA co-suppression and not necessarily to the production of the protein sequence.

In general, the virus sequence will be transferred in an appropriate cell or tissue culture of the

plant species using an *Agrobacterium* mediated transformation system or a direct gene transfer method according to the constraints of the tissue culture or cell culture method which can be successfully applied in a given species. A whole plant will be regenerated and the expression of the transgene will be characterized.

Though sugar beet is known as a recalcitrant species in cell culture, limiting the extent of practical genetic engineering applications in that species, there are number of isolated reports of successful transformation and regeneration of whole plants (38). A few examples of engineering tolerance to the BNYVV by transforming and expressing the BNYVV coat-protein sequence in the sugar beet genome have also been published (39, WO91/13159) though they rarely report data on whole functional transgenic sugar beet plants (40). In particular, reports show limited data on the level of resistance observed in infected conditions with transgenic sugar beet plants transformed with a gene encoding a BNYVV coat-protein sequence (41, 42).

A complete technology package including a sugar beet transformation method and the use of the expression of the BNYVV coat-protein sequence as resistance source in the transgenic sugar beet plant obtained by said transformation method has been described in the Patent Application WO91/13159.

Based on the information published, it can not be concluded that the coat-protein mediated resistance mechanism provides any potential for conferring to the sugar beet plant a total immunity to the BNYVV-infection by inhibiting completely the virus multiplication and diffusion mechanisms. To identify a resistance mechanism which significantly blocks the spread of the virus at the early stage of the infection process would be a major step toward successfully developing such a transgenic

resistance. In addition, such resistance would diversify the mechanisms of resistance available.

Because the disease is shown to expand in many countries or areas, at a speed depending upon the combination of numerous local environmental and agricultural factors, there is a strong interest diversifying genetic resistance mechanisms which may, alone or in combination, confer a stable and long lasting resistance strategy in the current and future varieties of sugar beet plants which are grown for industrial use.

The genome of beet necrotic yellow vein benyvirus (BNYVV) consists of five plus-sense RNAs, two of which (RNAs 1 and 2) encode functions essential for infection of all plants while the other three (RNAs 3, 4 and 5) are implicated in vector-mediated infections of host plants (*Beta macrocarpa*, *Beta vulgaris*, *Spinacear oleracea*, *Chenopodium quinoa*, etc.) roots (1). Cell-to-cell movement of BNYVV is governed by a set of three successive, slightly overlapping viral genes on RNA 2 known as the triple gene block (TGB) (2), which encode the viral proteins P42, P13 and P15 (gene products are designated by their calculated M_r in kilodalton (3).

In the following description, the TGB genes and the corresponding proteins will be identified by the following terms: TGB1, TGB2, TGB3 or by their encoded viral protein number P42, P13 and P15. TGB counterparts are present in other plant viruses and the characteristics of their TGB have allowed the classification of said viruses in two groups: the viruses of group I which include hordéiviruses, benyviruses, pecluviruses and pomoviruses and the viruses of group II represented by potexviruses and carlaviruses (4, 5, 6, 44).

For the viruses of group II, capsid protein is also involved in the cell-to-cell movement of viruses.

The development of a resistance to viral infections into a plant by blocking the cell-to-cell movement has been described for the potato viruses X (PVX) (45) and for the white clover mosaic virus (WClMV) (46) in *Nicotiana benthamiana*. These two viruses belong to the above-described group II. In both cases, various amino acids were replaced by Alanine in the hydrophilic part of the TGB sequence downstream of the N-terminal hydrophobic domain of said amino acid sequence. However, it was not possible with said mutants to obtain total resistance, especially when a virus challenger concentration is increasing into the plant.

Aims of the invention

The present invention aims to provide a new method for introducing various viral resistances into a cell and a plant and the viral resistant cell and plant obtained.

A main aim of the invention is to provide a new method for introducing BNYVV resistance into a cell and a plant and the BNYVV-resistant cell and plant, in particular a sugar beet cell and plant (*Beta vulgaris ssp.*), obtained.

Summary of the invention

The present invention provides the use of an alternative sequence of plant virus, especially the BNYVV, to obtain a high degree of tolerance to the viral infection, in particular to ensure a rapid and total blocking of virus multiplication and diffusion mechanisms in a plant, especially in the sugar beet plant (*Beta vulgaris*), including fodder beet, Swiss chard and table beet, which may also be subject to this viral infection. Expression of the resistance will be obtained in transgenic cell and plant, especially sugar beet cells and plants

produced by the transformation method subject to the Patent Application WO95/10178 or by other transformation methods based on *Agrobacterium tumefaciens* or direct gene transfer. Because of its high efficiency, the transformation method as described in WO95/10178 enables the production of large numbers of transformed plants, especially sugar beet plants, and will be preferred to develop transgenic plants which may be analysed and characterized for their level of viral resistance, especially BNYVV Resistance, including their field evaluation.

In the table 1 are represented viruses having a TGB2 sequence, the molecular weight of TGB2 of said viruses, their host and references.

Table 1

Virus	Size of TGB2 (kDa)	Host	Reference
GROUP I			
Beet necrotic yellow vein virus	13	beet	Bouzouba et al., *J. Gen. Virol.* 67, 1689-1700 (1986)
Barley stripe mosaic virus	14	barley	Gustafson et al., *Nucl. Acids Res.* 14, 3895-3909 (1986)
Potato mop top virus	13	potato	Scott et al., *J. Gen. Virol.* 75, 3561-3568 (1994)
Peanut clump virus	14	peanut	Herzog et al., *J. Gen. Virol.* 75, 3147-3155 (1994)
Beet soil-borne virus	13	sugar beet	Koenig et al., *Virology* 216, 202-207 (1996)
GROUP II			
Apple stem pitting virus	13	apple	Jelkman, *J. Gen. Virol.* 75, 1535-1542 (1994)
Blueberry scorch virus	12	blue-berry	Cavileer et al., *J. Gen. Virol.* 75, 711-720 (1994)
Potato virus M	12	potato	Zavriev et al., *J. Gen. Virol.* 72, 9-14 (1991)
White clover mosaic virus	13	clover	Forster et al., *Nucl. Acids Res.* 16, 291-303 (1988)
Cymbidium mosaic virus	14	orchid	Neo et al., *Plant Mol. Biol.* 18, 1027-1029 (1992)

The Inventors propose herewith a new method for providing resistance to plant viruses into a plant by blocking virus multiplication and diffusion mechanisms into said plant, especially into its root tissue. In order to demonstrate said resistance, the Inventors describe hereafter the effect of the overexpression of TGB2 sequence alone or in combination upon BNYVV multiplication and diffusion mechanism in plants of C. quinoa which are also

the hosts of the BNYVV virus and which could be more easily manipulated by the man skilled in the art.

It is known that BNYVV does not require synthesis of viral coat protein for production of local lesions on leaves of hosts such as *Chenopodium quinoa* (7), indicating that virion formation is not required for cell-to-cell movement.

However, the manner in which the TGB components assist in the movement process is not understood although computer-assisted sequence comparisons have detected characteristic conserved sequences which may provide clues to their function. Thus, the 5'-proximal TGB protein (TGB1) invariably contains a series of sequence motifs characteristic of an ATP/GTP-binding helicase while the second protein (TGB2) always has two potentially membrane-spanning hydrophobic domains separated by a hydrophilic sequence which contains a highly conserved peptide motif of unknown significance (6).

So far, no example has been reported of a virus of group I in which the three TGB members are arranged differently on the same RNA or are parcelled out to different genome RNAs, suggesting that their association in a particular order might be important in regulating their function.

The present invention concerns a method for inducing viral resistance to a virus of group I comprising the triple gene block (TGB2). Said viruses of group I comprise hordéiviruses, benyviruses, pecluviruses and pomoviruses, preferably viruses selected from the group consisting of the beet necrotic yellow vein virus, the barley stripe mosaic virus, the potato mop top virus, the peanut clump virus and the beet soil-borne virus; said method comprises the following steps:

- preparing a nucleotide construct comprising a nucleotide sequence corresponding to at least 70% of the wild-type

nucleotide sequence of TGB2 of said group I virus or its corresponding cDNA, being operably linked to one or more regulatory sequence(s) active in a plant,

- transforming a plant cell with the nucleotide construct, and possibly
- regenerating the transgenic plant from the transformed plant cell.

Advantageously, the nucleotide sequence corresponding to at least 70% of the wild-type nucleotide sequence of TGB2 or its corresponding cDNA comprise the substitution of at least one amino acid into another different amino acid in the TGB2 wild-type sequence SEQ ID NO. 1 (Fig. 1). Preferably, the substitution of at least one amino acid into another different amino acid is made in regions rich in hydrophilic amino acids usually present at the surface of the corresponding protein in its native configuration. Preferably, a modification is made in the hydrophilic region of the wild-type sequence downstream the N-terminal hydrophobic domain and just upstream the conserved central domain.

According to a preferred embodiment of the present invention, said amino acids are each substituted by the amino acid Alanine.

Preferably, the plant or plant cell is a plant or plant cell which may be infected by the above-described virus and is preferably selected from the group consisting of potato, barley, peanut and sugar beet.

The present invention concerns also the obtained plant cell and transgenic (or transformed) plant (made of said plant cells) resistant to said viruses and comprising said nucleotide construct.

The Inventors have also discovered unexpectedly that it is possible to induce BNYVV-resistance into a plant by a method which comprises the following steps:

- preparing a nucleotide construct comprising a nucleotide sequence corresponding to at least 70%, preferably at least 80%, more preferably at least 90%, of the wild-type nucleotide sequence comprised between the nucleotides 3287 and 3643 of the 5' strand of the genomic or subgenomic wild-type RNA 2 of the BNYVV or its corresponding cDNA, being operably linked to one or more regulatory sequence(s) active in a plant,
- transforming a plant cell with said construct, and possibly
- regenerating a transgenic plant from the transformed plant cell.

The nucleotide sequence comprised between the nucleotides 3287 and 3643 of the 5' strand of the genomic or subgenomic RNA 2 encoding the P13 protein is described in the Fig. 1 (SEQ ID NO. 1). A preferred mutated nucleotide sequence and its corresponding mutated amino acid sequence are described in the following specification as SEQ ID NO. 3 (Fig. 2).

Another aspect of the present invention concerns a plant cell and a transgenic plant (made of said plant cells) resistant to BNYVV and comprising a nucleotide construct having a nucleotide sequence corresponding to at least 70%, preferably at least 80%, more preferably at least 90%, of the nucleotide sequence comprised between the nucleotides 3287 and 3643 of the 5' strand of the genomic or subgenomic wild-type RNA 2 of BNYVV or its corresponding cDNA, being operably linked to one or more regulatory sequence(s) active in the plant.

Preferably, said plant cell or transgenic plant (made of said plant cells) resistant to BNYVV is obtained by the method according to the invention.

The variants of the wild-type nucleotide sequence (SEQ ID NO. 1) comprise insertion, substitution or deletion of nucleotides encoding the same or different

amino acid(s) (see Fig. 2). Therefore, the present invention concerns also said variants of the nucleotide sequence of SEQ ID NO. 1, for example SEQ ID NO. 3, which present at least 70%, preferably at least 80%, more preferably at least 90%, homology with said nucleotide sequence and which are preferably able to hybridise to said nucleotide sequence in stringent or non-stringent conditions as described by Sambrook et al., §§ 9.47-9.51 in *Molecular Cloning : A Laboratory Manual*, Cold Spring Harbor, Laboratory Press, Cold Spring Harbor, New York (1989).

A nucleotide sequence corresponding to at least 70%, preferably at least 80%, more preferably at least 90%, of the nucleotide sequence comprised between the nucleotides 3287 and 3643 of the 5' strand of the genomic or subgenomic wild-type RNA 2 of BNYVV or its corresponding cDNA, is preferably a sequence comprising a substitution of at least one amino acid into another different amino acid in the wild-type RNA2 sequence of the BNYVV or its corresponding cDNA. Preferably said substitution is made in regions in which hydrophilic amino acids are usually present at the surface of the protein in its native configuration (47) as described in Fig. 2 (A = substitution by Alanine). Preferably, said substitution of one or more amino acids is a mutation which allows the substitution of one or more amino acids into one or more Alanine amino acids.

According to a preferred embodiment of the present invention, said nucleotide sequence is SEQ ID NO. 3.

Preferably, said sequences are also able to induce BNYVV resistance into a plant.

The terms "induce a viral resistance into a plant" mean inducing a possible reduction or a significant delay into the appearance of infection symptoms, virus

multiplication or its diffusion mechanisms into the plant, especially in the root tissues.

In Fig. 3 are represented results showing the capacity of a plant coinoculated with virus containing a replicon construct with the nucleotide sequence according to the invention, especially the sequence SEQ ID NO. 3, to inhibit the movement by BNYVV in *C. Quinoa*. The infectious factor of BNYVV is shown by the appearance of local lesions of leaves of said plant after co-inoculation of wild-type virus S12. Fig. 3 presents the number of local lesions upon leaves of a plant by a BNYVV S12 isolate (comprising RNA1 and RNA2) when co-inoculated with various replicons incorporating either mutated sequences including SEQ ID NO. 3 identified in Fig. 2 or a wild-type nucleotide sequence (T).

Eight days after said inoculation, the local lesions are identified. The results of three experiments show that the decreasing of said effect is mostly observed with the co-inoculation of the mutated sequence SEQ ID NO. 3 (up to 100% inhibition). This effect is not due to a possible blocking effect upon RNA1 and RNA2 replication, but the replicons according to the invention allow a blocking of the biochemical mechanisms involved in cell-to-cell movements by the infectious virus.

The regulatory sequence(s) of the nucleotide sequence according to the invention are promoter sequence(s) and terminator sequence(s) active into a plant.

The nucleotide construct may also include a selectable marker gene, which could be used to identify the transformed cell or plant and express the nucleotide construct according to the invention.

Preferably, the cell is a stomatal cell and the plant is a sugar beet (*Beta vulgaris ssp.*) made of said cells.

According to the invention, the promoter sequence is a constitutive or foreigner promoter sequence. Examples are 35S Cauliflower Mosaic Virus promoter sequence, polyubiquitin *Arabidopsis thaliana* promoter (43), a promoter which is mainly active in root tissues such as the *par* promoter of the haemoglobin gene from *Perosponia andersonii* (Landsman et al., Mol. Gen. Genet. 214 : 68-73 (1988)) or a mixture thereof.

A last aspect of the present invention is related to a transgenic plant tissue such as fruit, stem, root, tuber, seed of the transgenic plant according to the invention or a reproducible structure (preferably selected from the group consisting of calluses, buds or embryos) obtained from the transgenic plant or the cell according to the invention.

The techniques of plant transformation, tissue culture and regeneration used in the method according to the invention are the ones well known by the person skilled in the art. Such techniques are preferably the ones described in the International Patent Applications WO95/10178 or WO91/13159 corresponding to the European Patent Application EP-B-0517833, which are incorporated herein by reference. These techniques are preferably used for the preparation of transgenic sugar beets according to the invention.

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